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BIOCHEMICAL STUDIES ON THE MECHANISM OF DRUG ACTION

SEMIANNUAL REPORT

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PREFACE

The following is a semiannual report for Grant No. DAHC 19-71-G-0002 (Army Project Number 2N061102B71D) and represents detailed results and discussion of work accomplished from August 1, 1971 to January 31, 1972.

This report deals with the mode of action of 5,5'-diphenyl-2-thiohydantoin (DPTH) and the effect of aspirin on gluconeogenic enzymes.

The author wishes to thank the U. S. Army Research Office for their support of this project. The close association and contributions of Dr Eugene M. Sporn, Chief, Special Projects Branch, Life Sciences Division, with this research is acknowledged.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animals, Resources, National Academy of Sciences, National Research Council.

ABSTRACT

1. The effects of 5,5'diphenyl-2-thiohydantoin (DPTH) on mitochondria were examined *in vitro* and *in vivo*, on well delineated sites known to be profoundly affected by thyroid hormones, in order to test the hypothesis that thyroid hormones regulate metabolic responses through mitochondrial and cytosolic processes. The effect of methimidazole, another antithyroid drug, was studied *in vitro* in parallel systems. Addition of DPTH greatly decreased the capacity of mitochondria to synthesize precursors for gluconeogenesis as a result of the inhibition of pyruvate carboxylation. DPTH was found to inhibit α-glycerophosphate dehydrogenase, the enzyme involved in the regulation of hydrogen transfer to cytochromes through the cytosolic and mitochondrial α-glycerophosphate cycle. DPTH also inhibited β-hydroxybutyrate dehydrogenase. The state 3 respiration with pyruvate, α-ketoglutarate, succinate and α-glycerophosphate was inhibited by DPTH. Methimidazole has no effect in blocking the peripheral action of thyroxine and was non-inhibitory in the above systems studied. *In vivo* experiments where DPTH was added to the diets, a large inhibition of liver gluconeogenic enzyme activities, α-glycerophosphate dehydrogenase and oxygen consumption by rat liver tissue slices were observed.

2. Rats fed a diet containing 0.25% acetylsalicylic acid for a period of 5 weeks grew normally and did not exhibit symptoms characteristic of toxicity. Acetylsalicylic acid feeding decreased the concentration of glycogen in liver, the activities of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in liver, and the activity of phosphoenolpyruvate carboxykinase in kidney. The activities of glucose-6-phosphatase and fructose-1,6-diphosphatase in liver and pyruvate carboxylase in kidney were not affected. Intraperitoneal injection of acetylsalicylate (5 mg/100 g body wt.) to control rats and in vitro addition of acetylsalicylate (5mM) to the enzyme assays did not inhibit any of the tested enzymes. The findings suggest that chronic acetylsalicylic acid ingestion may inhibit gluconeogeneisis by decreasing the activities of the regulatory enzymes.

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INTRODUCTION

The objectives of this research project were two-fold: 1) to examine both the *in vitro* and *in vivo* effects of 5,5'-diphenyl-2-thiohydantoin (DPTH) on the sites known to be affected by thyroid hormone and; 2) to investigate the effects of acetylsalicylic acid on gluconeogenesis.

It has been established that thyroid hormone affects carbohydrate metabolism (1-5) and the metabolic rate in liver, kidney, heart, skeletal muscle and other tissues (6-10). The oxygen consumption in these tissues has been reported to be markedly increased. Thyroxine also had a highly specific (10-13) effect on mitochondrial α-glycerophosphate dehydrogenase: a large increase in its activity was observed in thyroxine treated animals. The physiological importance of this stimulation of α-glycerophosphate dehydrogenase activity by thyroxine may be in its ability to act as a carrier of cytoplasmic reducing equivalents to mitochondrial cytochrome carriers (14-17). The effect of this hormone is to lower the extramitochondrial NADH/NAD^+ ratio which can then stimulate carbohydrate and glycogen catabolism in cytosol as suggested by Lee and Lardy (10).

Ruegamer* has screened over sixty compounds for possible anti-thyroid activity in the rat. One of these compounds, 5,5'-diphenyl-2-thiohydantoin (DPTH) was found to be a very potent inhibitor of thyroxine stimulated metabolic responses.

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* W. R. Ruegamer, unpublished observation.

5,5'-diphenyl-2-thiohydantoin at 0.06% of the diet totally inhibited metabolic responses to thyroid hormone (20 mg per Kg).

Administration of salicylates to diabetic (18-20) and fasted normal humans (20), to experimental animals with endocrine inbalances (21-23), and to fasted normal rats (24, 25) causes profound decreases in blood glucose concentration. This hypoglycemic effect may be mediated through inhibition of gluconeogenesis, stimulation of glucose consumption or by a combination of both mechanisms.

EXPERIMENTAL

A. Methods

Animals and Diets

Male albino rats (Sprague-Dawley strain) weighing 80-100 g kept in individual cages with wire-mesh screen bottoms, were used in all experiments. In Study I, the control group was fed a ground Purina chow diet and the test group Purina Chow diets to which 5,5'-diphenyl-2-thiohydantoin (DPTH) was added in amounts described in the legends to the tables. There were no differences in body weight gain or food consumption between control and DPTH fed rats. In Study II, the control group was also fed a ground Purina Chow diet and the test group Purina Chow + 0.25% acetylsalicylic acid. Each rat consumed 10-20 g of food/day and therefore, 25-50 mg of acetylsalicylic acid/day. At this dose, toxicity was not evident and the food consumptions and growth rates for

both groups of rats were not significantly different. All animals had free access to tap water.

Mitochondrial Experiments

All experiments were performed with intact rat liver mitochondria, isolated according to the method of Schneider (26), except that the mitochondria were centrifuged at 10,000 x g for 10 minutes. The incubation, processing of samples, analyses for pyruvate, malate, α-glycerophosphate, dihydroxyacetone phosphate, citrate, $^{14}CO_2$ incorporated, acetoacetate (AcAc), β-hydroxybutyrate (β-OH) and mitochondria nitrogen were performed according to methods previously cited (27, 28). Oxygen utilization, ADP/oxygen ratios and respiratory control indices were determined by the method of Chance and Williams (29) using an Oxygraph (Gilson Medical Electronics).

Measurement of Oxygen Consumption by Tissue Slices

The liver was quickly removed and cooled in Krebs-Ringer's phosphate media on ice. Slices of liver were prepared free-hand, blotted on moist filter paper to remove excess solution and weighed in a torsion balance. The slices were then placed directly in Warburg flasks containing 3 ml of Krebs-Ringer phosphate solution with 10 mM glucose as substrate. Flasks were placed in 37°C water bath. shaken and gassed with 100% oxygen for a 5 minute period. Side arms were then closed and measurements of oxygen consumption were commenced at 15 minutes after placing the flasks in the water bath. Standard manometric techniques and calculations were utilized to measure oxygen consumption of the slices and the results are expressed as μmoles of oxygen per gram of dry tissue per minute.

Enzyme Assays

Rats were killed by decapitation and liver was removed rapidly, weighed in prepared beakers containing cold media (0.25 M sucrose, 0.02 M TRIS, pH 7.3, 0.001 M EDTA, and 0.001 M reduced glutathione). Homogenates were adjusted to 10% w/v of liver.

Pyruvate carboxylase (E.C.6.5.1.1), phosphoenolpyruvate carboxykinase (E.C.4.1.1.32), fructose-1,6-diphosphatase (E.C. 3.1.3.11) and glucose-6-phosphatase (E.C.3.1.3.9) were assayed by previously cited methods (30).

RESULTS AND DISCUSSION

Influence of 5,5'-Diphenyl-2-thiohydantoin (DPTH) and Methimidazole (MMI) on α-Glycerophosphate and L-Octanoylcarnitine Metabolism in Rat Liver Mitochondria

It has been shown that administration of thyroxine to rats caused an increase in mitochondrial α-glycerophosphate dehydrogenase (10, 17). When mitochondria from normal rat livers were incubated with DPTH, the conversion of α-glycerophosphate to dihydroxyacetone-phosphate was inhibited (Table I). Six-tenths millimolar DPTH inhibited α-glycerophosphate conversion by 81%. However, methimidazole had no inhibitory effect.

Results in Table II show the effect of varied DPTH concentrations on L-octanoylcarnitine metabolism to ketone bodies in the absence of any additional added substrate. It can be seen that DPTH strongly inhibits formation of total ketone bodies and also inhibits conversion of AcAc to β-OH butyric acid. The degree of inhibition of fatty acid oxidation is dependent on the concentration of DPTH in the incubation media.

Inhibition by DPTH of L-Octanoylcarnitine Oxidation in Disrupted Rat Liver Mitochondria

Since fatty acid oxidizing enzyme systems are located in the mitochondrial membrane (32), it was possible that DPTH blocked membrane sites that were involved in oxidation of fatty acids. Results in Table III show the effect of 1.2 mM DPTH on mitochondrial oxidation of fatty acids in intact, sonicated, frozen-thawed and digitonin treated mitochondria. It is clear that total ketone body production was markedly decreased in the presence of DPTH in intact mitochondria as well as in fragments formed by sonication, freeze-thawing or digitonin treatment.

At this high concentration of DPTH (1.2 mM), even acetoacetate formation was greatly decreased, from 20.96 to 7.81 nmoles N/ minute. The ratio of β-hydroxybutyrate to acetoacetate in intact mitochondria in the presence of DPTH was also decreased. Sonication or treatment of mitochondria with digitonin greatly altered the β-hydroxybutyrate to acetoacetate ratios. In the presence of 1.2 mM DPTH, total amount of ketones formed was so small that the ratio of β-OH to AcAc which was reported is probably unreliable under these circumstances. In frozen-thawed mitochondria the ratio of β-OH/AcAc was also decreased. This ratio obtained with frozen-thawed mitochondria in the presence of DPTH is probably reliable since total amounts of ketones formed are several times larger than those with sonicated or digitonin treated mitochondria. These experiments indicate that DPTH inhibition of fatty acid oxidation is not a membrane dependent phenomenon.

Effect of DPTH on Synthesis of Precursors for Gluconeogenesis by Rat Liver Mitochondria

The utilization of pyruvate and the formation of malate and citrate and the incorporation of $^{14}CO_2$ into organic acids by intact liver mitochondria in the presence of DPTH, MMI and L-octanoylcarnitine (OC) are shown in Table IV.

The major organic acids accumulated by isolated rat liver mitochondria are malate, citrate and fumarate. L-octanoylcarnitine (0.7 mM) added to incubation media decreased pyruvate utilization and decreased the ratio of pyruvate used to $^{14}CO_2$ incorporated. This decrease in pyruvate utilization in the presence of L-octanoylcarnitine was a result of competition of pyruvate oxidase and the fatty acid oxidase system for CoA (27, 33). Addition of 0.6 mM DPTH decreased pyruvate utilization from 545.8 to 379.1 nmoles $^{14}CO_2$ incorporation from 206.2 to 89.5 nmoles and increased the ratio of pyruvate used to $^{14}CO_2$ incorporated from 2.6 to 4.3.

Addition of 0.6 mM DPTH and 0.7 mM octanoylcarnitine (OC) further decreased pyruvate utilization from 395.8 nmoles (in the presence of OC alone) to 291.2 nmoles. The $^{14}CO_2$ incorporation was 89.5 nmoles, the same as with DPTH alone. The effect of MMI with and without OC on pyruvate metabolism was minimal.

The estimation of pyruvate carboxylation based on $^{14}CO_2$ incorporation is minimal because labeled $^{14}CO_2$ may be lost by oxidative decarboxylation during forward metabolism in the TCA cycle. In addition, unlabeled CO_2 produced from oxidation of pyruvate (28) may be fixed into organic acids. Thus, the observed changes

in $^{14}CO_2$ incorporation may be influenced by the rates of pyruvate oxidation and by the rates of TCA cycle decarboxylation reactions. A more accurate way to estimate the amount of pyruvate carboxylated is by summing all products synthesized (malate, citrate, fumarate and succinate) as previously described (32). When pyruvate carboxylase activity was estimated by summing up total products formed, DPTH inhibited carboxylation by 43% in the absence of OC and by 47% in the presence of OC.

Effect of Succinate and 2,4-Dinitrophenol (DNP) on DPTH Inhibition of L-Octanoylcarnitine Oxidation by Rat Liver Mitochondria

Table V shows the effects of succinate and DNP on ketone body accumulation from fatty acid oxidation, in the presence of pyruvate. DPTH significantly inhibited ketone accumulation (187.38 to 52.41 umoles ($P<0.01$) and decreased β-OH/AcAc ratios from 1.47 to 0.60 ($P<0.001$). 6.6 mM succinate in the absence of DPTH did alter significantly ketone body accumulation. However the redox ratio was significantly ($P<0.001$) increased (1.47 to 2.65). In the presence of DNP there was a large increase in the amount of acetoacetate found ($P<0.001$) and a decrease in the redox ratio from 1.47 to 0.36. However, the total ketone body accumulation did not differ from controls. Addition of succinate with DPTH did not reverse the DPTH inhibition of OC oxidation to ketones. In the presence of both DPTH and DNP, the total ketones found were higher than with DPTH alone but not so high as control group A. The redox state ratio remained low. The combination of succinate, DPTH and DNP suppressed the total ketones formed by 84%.

Effect of DPTH on Respiration and Oxidative Phosphorylation of Pyruvate, α-Ketoglutarate, Succinate and α-Glycerophosphate by Rat Liver Mitochondria

Table VI illustrates the effect of DPTH on respiration of rat liver mitochondria in the presence of various substrates. The State 3* respiration rates were significantly decreased with all four substrates studied. The respiratory rate in State 4* was significantly decreased with α-KG as substrate ($P<0.005$) in the presence of DPTH, whereas with pyruvate, succinate and α-glycerophosphate there was no significant difference. Highly significant decreases were observed in the respiratory control ratios with all substrates (Table VI). The ratio of ADP/oxygen was slightly but significantly decreased with all substrates in the presence of DPTH (Table VI).

Results in Table VII show the State 3* respiration rates and ratio of ADP/oxygen* in the presence of DPTH with and without added pentabarbitol. With pyruvate as substrate, pentabarbitol caused no further decrease in State 3 oxygen consumption than did DPTH. Pentabarbitol did not inhibit succinate oxidation as DPTH did. With or without pentabarbitol in the system, the ratio of ADP/oxygen was significantly lower in the presence of DPTH with pyruvate as substrate.

*State 3 is the condition in which all required components are present and the respiratory chain itself is the rate-limiting factor. State 4 is the condition in which only ADP is lacking. The ratio of State 3 to State 4 is defined as the respiratory control index. This ratio is a more sensitive criterion of the intactness of mitochondrial structure than a high phosphorus/oxygen ratio (36). The ADP/O ratio is equivalent to phosphorus/oxygen ratio.

Effect of DPTH on α-Glycerophosphate Metabolism by Rat Liver Mitochondria

Table VIII shows that mitochondria isolated from rats fed dietary DPTH for a total of 39 days almost completely inhibited the conversion of α-glycerophosphate to dihydroxyacetone phosphate. This inhibitory effect by DPTH *in vivo* is in good agreement with results obtained *in vitro* in Table I.

Inhibition of Oxygen Consumption by Rat Liver Tissue Slices from DPTH Fed Rats

Results in Table IX show that there is a large decrease in oxygen consumption from 4.21 ± 0.29 to 2.51 ± 0.15 μmole O_2 dry weight/minute by liver tissue slices of DPTH treated rats. This decrease in oxygen consumption by tissues from DPTH treated animals may be due in part to decrease in respiration as seen in Table VI where DPTH was added to rat liver mitochondria.

The metabolic changes that are evoked by thyroid hormones are associated with the electron transport system, reactions in the tricarboxylic acid cycle and enzyme systems associated with generation and the transport of reducing equivalents between mitochondria and cytosol.

In the present study we have examined the effect(s) of 5,5'-diphenyl-2-thiohydantoin (DPTH) and methimidazole (MMI) on sites in mitochondria that are known to be affected by thyroid hormones. MMI had no *in vivo* effect in blocking the peripheral action of thyroxine*, and was used in our studies to demonstrate that the

*W. R. Ruegamer, unpublished observations.

compound was also inactive in the in vi o systems in comparison with DPTH, which has strong inhibiting effects.

Menahan and Wieland (34) have shown that in hyperthyroid rats there was a large decrease in the activities of two key rate limiting gluconeogenic enzymes: pyruvate carboxylase and phosphoenolpyruvate carboxykinase (35). Treatment of hypothyroid rats with thyroid hormones greatly increased the activities of pyruvate carboxylase and phosphoenolpyruvate carboxykinase 3, 36, 38). In our studies (Table X), we have also noted that feeding animals 0.10% DPTH diets for two weeks resulted in a large, significant decrease ($P<0.001$) in pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities in addition to total inhibition of mitochondrial α-glycerophosphate dehydrogenase activity (Table VIII) after 39 days of feeding of DPTH. The inhibition of these enzymes by DPTH is consistent with its peripheral antithyroid activity.

Addition of DPTH in vitro greatly decreased mitochondrial synthesis of gluconeogenic precursors as shown in Table 4 where carboxylation of pyruvate to dicarboxylic acids was markedly inhibited by DPTH.

Thyroid hormone may regulate hydrogen transfer to the cytochromes by acting on cytosolic and mitochondrial α-glycerophosphate cycle. This was proposed by Bücher and Klingenberg (16) and Estabrook and Sacktor (13). In our studies α-glycerophosphate dehydrogenase was strongly inhibited by DPTH in vitro (Table I) and in vivo (Table VIII).

The inhibition of fatty acid oxidation by DPTH, possibly at the β-hydroxybutyrate dehydrogenase step appears to be an effect not related to its antithyroid properties since the rate of β-hydroxybutyrate oxidation decreased during thyroid hormone treatment while the dehydrogenase activity remained unchanged (10). This aspect requires further investigation. Perhaps these observations indicate a new site for thyroxine action.

The stimulation of oxidative metabolism as represented by increased oxygen consumption is a characteristic of thyroid hormone administration (6-10, 15). It has been suggested that stimulation of mitochondrial respiration and basal metabolic rate by thyroid hormone is not the result of uncoupling of oxidative phosphorylation or of mitochondrial structural changes, but is possibly related to synthesis of respiratory chain components (38-43). Thus, inhibition of State 3 respiration with pyruvate, α-ketoglutarate, succinate and α-glycerophosphate by DPTH as shown in Table VI is consistent with the antithyroid effects of this compound in accord with observations of Bronk (44) and Volfin (43) which showed that both State 3 and State 4 respirations were greatly decreased in thyroidectomized animals.

The uncoupling of oxidative phosphorylation by DNP was significantly lower in the presence of DPTH. This suggests that DPTH may exert its inhibitory effect within the respiratory chain. We have shown that DPTH is a potent inhibitor (Table II) of NAD-linked β-hydroxybutyrate dehydrogenase reaction and that it also inhibits respiration with both NAD^+ or FAD^+ linked substrates. It can be seen in Table VII that with pyruvate as substrate in the

presence of pentabarbitol, DPTH did not further inhibit State 3 respiration, but the ADP/O ratio (Table VII) was decreased to a larger degree with both pentabarbitol and DPTH in the system. In the presence of both pentabarbitol and DPTH, State 3 respiration of succinate was inhibited to the same extent as with DPTH alone while the ADP/oxygen ratio was decreased in the presence of DPTH but not pentabarbitol. From these studies it can be concluded that DPTH may interact at more than one phosphorylation site in the respiratory chain.

Activities of Gluconeogenic Enzymes in Acetylsalicylic Acid-Fed and Control Rats

After feeding the control and acetylsalicylic acid-containing diets for 5 weeks, rats were fasted for 20 hours prior to sacrifice in order to stimulate gluconeogenesis (45) and to increase the activities of the "gluconeogenic enzymes* (45-48). The activities of the "gluconeogenic enzymes" from liver are shown in Table XI. In kidney (Table XII), phosphoenolpyruvate carboxykinase was significantly reduced by acetylsalicylic acid feeding. Pyruvate carboxylase activity was only slightly reduced, however, and the difference between the control and acetylsalicylic acid fed groups was not significant.

Intraperitoneal administration of acetylsalicylate (5 mg/100 g body wt) to control rats 4 hours prior to sacrifice and addition of 5 mM acetylsalicylate to the assay media failed to affect the

*These are the four enzymic reactions by which the cell probably regulates the process of gluconeogenesis; ie.e., pyruvate carboxylase, EC.6.4.1.1; phosphoenolpyruvate carboxyinase, EC.4.1.1.32; fructose-1,6-diphosphatase, EC.3.1.3.11; glucose-6-phosphatase, EC.3.1.3.((46).

activities of the "gluconeogenic enzymes".*

Effect of Acetylsalicylic Acid Feeding on Liver Glycogen Levels

Liver glycogen levels are reported in Table XIII. Acetylsalicylic acid feeding caused a significant reduction in the concentration of liver glycogen. This effect is consistent with the work of Vaughan et al. (25), which also showed that chronic acetylsalicylic acid ingestion leads to decreased liver glycogen.

Salicylates can produce a hypoglycemic effect in experimental animals (21-25) and humans (18-20). However, the mechanisms by which the effect is produced have not been elucidated. It is apparent that hypoglycemic activity must involve increased glucose utilization, decreased glucose synthesis or decreased glucose uptake through the intestinal tract. Fortunately one possibility, the latter, appears to have been eliminated by experiments which demonstrated salicylate hypoglycemia during starvation (20, 25, 49, 50) and other experiments which showed that salicylates do not diminish the in vivo rate of glucose absorption by the intestinal tract of animals (51, 52) and humans (20, 53).

A considerable amount of evidence has accumulated which implicates both decreased gluconeogenesis and increased glucose utilization. Both mechanisms may be affected simultaneously by uncoupling of oxidative phosphorylation. Salicylates are known to cause uncoupling in vitro (54, 55) and this effect has been related to increased oxygen and food consumption in vivo (54). Uncoupling could limit the amount of cellular energy available for

*In the assay for fructose-1,6-diphosphatase (9), a high activity of glucose-6-phosphate dehydrogenase was used, thus preventing inhibition at this point by the acetylsalicylate. Salicylates inhibit dehydrogenases (see discussion).

glucose synthesis. Gluconeogenesis is an anabolic process which requires the input of considerable amounts of energy; for example, a minimum of 6 moles of ATP are required to produce 1 mole of glucose from 2 moles of lactate.

Previous reports have also indicated that salicylates inhibit gluconeogenesis and increase glucose utilization by causing a fall in blood non-esterified free fatty acid (FFA) levels (56-59). FFA have been shown to stimulate gluconeogenesis in liver perfusions (60-62), kidney slices (63) and intact animals (64). In addition, high concentrations of plasma FFA can decrease tissue utilization of glucose (56). Thus, agents which lower plasma FFA would be expected to lower blood glucose levels as well (58).

The possibility that gluconeogenesis may be regulated by salicylate effects on enzyme activities has not been adequately explored. Reports have shown that salicylates can inhibit many enzymes in vitro (65-67), notably the dehydrogenases by competing for pyridine nucleotides (67), but the relationships between these inhibitions and specific effects on gluconeogenesis are obscure.

In the present report, we have shown that key regulatory enzymes of gluconeogenesis, i.e., pyruvate carboxylase and phosphoenolpyruvate carboxykinase in liver and phosphoenolpyruvate carboxykinase in kidney are significantly inhibited by chronic acetylsalicylic acid administration. These results are in agreement with those of Vaughan et al. (25) which showed decreased blood glucose concentrations accompanied by low glycogen levels

in liver after chronic acetylsalicylic acid ingestion. Our results imply that glucose synthesis via gluconeogenesis may be impaired by chronic acetylsalicylic acid ingestion. The precise mechanism by which acetylsalicylic acid inhibited the enzyme activities is not known but probably involved increased enzyme degradation and/or decreased enzyme synthesis. Direct inhibition of active enzyme was probably not a factor, however, since neither acute administration of acetylsalicylate to control rats nor addition of acetylsalicylate *in vitro* to the enzyme assays produced inhibitory effects.

Further work in this area is anticipated and we are now attempting to relate decreased gluconeogenic enzyme activities to decreased rates of glucose synthesis.

D. Publications Supported by This Grant

1972 - Mode of Action of 5,5'-Diphenyl-2-thiohydantoin (DPTH): Sites of Action of DPTH in Mitochondria in Relationship to Thyroxine Stimulated Responses. M. A. Mehlman, R. B. Tobin, W. R. Ruegamer, and M. M. Madappally. Biochem. Pharmacol. In press.

1972 - The Inhibitory Effects of Acetylsalicylic Acid Feeding on Gluconeogenic Enzymes in Rat Liver and Kidney. M. M. Madappally, C. R. Mackerer, and M. A. Mehlman. Life Sciences 11 (Part II), 77.

The complete list of publications will be included with the annual report, July, 1972.

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TABLE I

EFFECT OF 5,5 DIPHENYL-2-THIOHYDANTOIN (DPTH) AND METHIMIDAZOLE (MMI) ON α-GLYCEROPHOSPHATE METABOLISM IN RAT LIVER MITOCHONDRIA

mM Addition to System	Metabolite Changes in μmoles/mg N/minute	
	α-Glycerophosphate used	Dihydroxyacetonephosphate found
None	75.0	63.3
0.3 DPTH	31.3	21.0
0.6 DPTH	14.6	10.2
1.46 MMI	77.1	61.5

The reaction mixture contained 3.3 mM ATP, 10 mM $MgSO_4$, 13.3 mM $KHCO_3$, 13.3 mM potassium phosphate and 13.3 mM potassium triethanolamine buffers pH 7.3, 5 mg of fatty acid poor albumin, and 20 mM α-Glycerophosphate. Mitochondria from 0.5 g of liver were suspended in 0.5 ml of 0.25 M sucrose and contained 2.4 mg of nitrogen. The final volume was 3.0 ml adjusted with 0.7 to 1.2 ml of 0.154 M KCl. The incubation time was 12 minutes.

TABLE II

METABOLISM OF L-OCTANOYLCARNITINE BY LIVER MITOCHONDRIA IN THE PRESENCE OF VARIED CONCENTRATIONS OF 5,5-DIPHENYL-2-THIOHYDANTOIN

Addition to System	Metabolite Changes in nmoles/ mg N/ minute			
	Acetoacetate found	β-Hydroxybutyrate found	Total Ketones	Ratio of β-Hydroxybutyrate / Acetoacetate
mM DPTH				
None	41.14	104.68	145.82	2.54
	40.10	94.27	134.37	2.35
0.15	35.93	84.63	120.56	2.35
	36.71	82.03	118.74	2.23
0.30	45.57	8.85	54.42	0.19
	46.35	8.07	54.42	0.17
0.60	36.45	4.94	41.30	0.13
	36.71	5.20	41.91	0.14
0.90	28.38	3.90	32.28	0.13
	23.43	4.68	28.11	0.20

The reaction mixture contained 3.3 mM ATP, 10 mM $MgSO_4$, 13.3 mM $KHCO_3$, 13.3 mM potassium phosphate and 13.3 mM potassium triethanolamine buffers pH 7.3, 5 mg of fatty acid poor albumin, and 1.0 mM L-Octanoylcarnitine. Mitochondria from 0.5 g of liver were suspended in 0.5 ml of 0.25 M sucrose and contained 3.2 mg of nitrogen. The final volume was 3.0 ml adjusted with 0.7 to 1.2 ml of 0.154 M KCl. The incubation time was 12 minutes.

TABLE III

METABOLISM OF L-OCTANOYLCARNITINE IN INTACT, SONICATED, FROZEN-THAWED AND DIGITONIN TREATED RAT LIVER MITOCHONDRIA IN THE PRESENCE OF 5,5 DIPHENYL-2-THIOHYDANTOIN (DPTH)

Addition to System and Treatment	Metabolite Changes in nmoles/mg/minute			
	Acetoacetate found	β-Hydroxybutyrate found	Total Ketones	Ratio β-Hydroxybutyrate/AcAc
mM				
Intact - None	20.96	38.75	59.71	1.84
	24.06	45.62	69.68	1.89
1.2 DPTH	7.81	4.18	11.99	0.53
	5.62	3.18	8.80	0.56
Sonicated[a] - None	21.34	5.18	26.52	0.24
	21.50	5.50	27.00	0.26
1.2 DPTH	0.96	1.56	2.52	1.61
	0.68	1.56	2.24	2.30
Frozen thawed[b] - None	23.62	22.90	46.52	0.97
	24.53	20.40	44.93	0.83
1.2 DPTH	6.40	3.09	9.49	0.49
	4.84	3.93	8.77	0.88
Digitonin[c] - None	20.68	2.81	22.49	0.13
	16.84	3.68	20.52	0.21
1.2 DPTH	1.96	0.81	2.77	0.41
	1.31	0.50	1.81	0.38

[a]Sonicated for 90 seconds.

[b]Frozen for 20 minutes and thawed under running cold water.

[c]Digitonin was added at 0.05% weight/volume to mitochondria, for 5 minutes before incubation.

The reaction mixture was the same as described in Table 2 except that 0.67 mM octanoylcarnitine was used as substrate.

TABLE IV

EFFECT OF 5,5 DIPHENYL-2-THIOHYDANTOIN (DPTH) AND METHIMIDAZOLE (MMI) ON PYRUVATE METABOLISM BY RAT LIVER MITOCHONDRIA

Addition to System mM	Metabolite Changes in nmoles/mg/min				
	Pyruvate used	$^{14}CO_2$ Incorp.	Malate found	Citrate found	Ratio $\frac{\text{Pyruvate used}}{^{14}CO_2 \text{ Incorp.}}$
None	545.8	206.2	131.2	85.4	2.6
0.7 OC	395.8	172.9	137.5	58.3	2.2
0.6 DPTH	379.1	89.5	81.2	43.7	4.3
0.6 DPTH, 0.7 OC	291.2	89.5	66.6	33.3	3.1
1.43 MMI	525.0	175.0	116.6	91.6	3.0
1.43 MMI, 0.7 OC	420.8	185.4	135.4	64.5	2.2

L-Octanoylcarnitine (OC)

The reaction mixture contained 10 mM pyruvate, 3.3 mM ATP, 10 mM $MgSO_4$, 13.3 mM $KH^{14}CO_3$, 13.3 mM potassium phosphate and 13.3 mM potassium triethanolamine buffers, pH 7.3, 5 mg of fatty acid poor albumin and 1.0 mM L-Octanoylcarnitine. Mitochondria from 0.5 g of liver were suspended in 0.5 ml of 0.25 M sucrose and contained 3.2 mg of nitrogen. The final volume was 3.0 ml adjusted with 0.7 to 1.2 ml of 0.154 M KCl. The incubation time was 15 minutes.

TABLE V

EFFECT OF 5,5 DIPHENYL-2-THIOHYDANTOIN, SUCCINATE AND 2,4 DINITROPHENOL (DNP) ON FATTY ACID METABOLISM IN RAT LIVER MITOCHONDRIA

Addition to System	METABOLITE CHANGES			
	Acetoacetate found	β-Hydroxybutyrate found	Total Ketones found	Ratio - $\frac{\beta\text{-Hydroxybutyrate}}{\text{Acetoacetate}}$
	nmoles/mg/min			
None	78.47±10.74	108.91± .58	187.38±11.08	1.47± .20
0.6 DPTH	32.83± 1.37	19.58±1.16	52.41± .10	.60± .06
6.6 Succinate	46.10± .46	122.22±2.31	168.32± 2.37	2.65± .06
0.1 DNP	129.44± 3.33	46.10±2.30	175.54± 5.31	.36± .01
0.6 DPTH, 6.6 Succinate	13.24± 1.09	19.01± .58	32.25± 1.08	1.21± .23
0.6 DPTH, 0.1 DNP	67.98± 1.82	28.83±2.74	96.81± 1.24	.43± .05
0.1 DPTH, 0.1 DNP, 6.6 Succinate	10.94± .74	19.58± .67	30.52± 1.37	1.56± .31

The reaction mixture contained 8.4 mM pyruvate, 3.3 mM ATP, 10 mM $MgSO_4$, 13.3 mM $KHCO_3$, 13.3 mM potassium phosphate and 13.3 mM potassium triethanolamine buffers pH 7.3, 1 mM L-octanoylcarnitine and 5 mg of fatty acid poor albumin. Mitochondria from 0.5 g liver were suspended in 0.25 M sucrose and contained 2.4 mg nitrogen. The final volume was 3.0 ml adjusted with 0.7 to 1.2 ml of 0.15 M KCl. Mitochondria from four different animals. All values are means ± S.E.M. The significance was determined by student's t test. Means not followed by the same letter are significantly different. $P<0.025$.

TABLE VI

RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF SUBSTRATES BY RAT LIVER MITOCHONDRIA

IN THE PRESENCE OF 5,5 DIPHENYL-2-THIOLYDANTOIN (DPTH)

Substrate (mM)	STATE 3 Qo_1 (mumoles O_2/mg P/minute) Without DPTH	STATE 3 With DPTH	STATE 4 Qo_2 (mumoles O_2/mg P/minute) Without DPTH	STATE 4 With DPTH	RATIO STATE 3/STATE 4 Without DPTH	RATIO STATE 3/STATE 4 With DPTH	RATIO ADP/O Without DPTH	RATIO ADP/O With DPTH	(DNP 0.1 mM) Qo_2 (mumoles O_2/mg P/minutes) Without DPTH	(DNP 0.1 mM) Qo_2 (mumoles O_2/mg P/minutes) With DPTH
10 Pyruvate	34.7±2.6	20.3±1.8	8.7±0.7	8.3±0.2	4.10±0.15	2.36±0.25	2.30±0.02	2.04±0.05	40.3±3.3	22.8±2.7
	(P<0.005)		(N.S.)		(P<0.001)		(P<0.05)		(P<0.005)	
10 α-Ketoglutarate	25.0±0.3	8.7±0.6	4.7±0.2	3.2±0.2	5.51±0.18	2.95±3.5	2.81±0.03	2.08±0.07	25.5±0.3	9.7±0.5
	(P<0.001)		(P<0.005)		(P<0.001)		(P<0.001)		(P<0.005)	
60 Succinate	82.0±3.8	52.5±2.5	29.3±1.7	24.2±2.2	2.85±0.24	2.23±0.16	1.57±0.03	1.35±0.02	78.5±6.6	62.4±3.7
	(P<0.001)		(N.S)		(P<0.001)		(P<0.005)		(P<0.05)	
15 α-Glycerophosphate	26.8±2.1	21.5±1.0	9.5±0.5	10.5±0.5	2.81±0.19	2.08±0.09	2.24±0.04	1.96±0.07	23.4±1.1	19.5±0.5
	(P<0.05)		(N.S.)		(P<0.005)		(P<0.025)		(P<0.05)	

The reaction mixture contained pyruvate 10 mM plus malate 1 mM, or succinate 60 mM or α-ketoglutarate 10 mM or α-glycerophosphate 15 mM, TRIS buffer 20 mM, EDTA 1 mM, KCl 15 mM, $MgCl_2$ 5 mM, potassium phosphate 30 mM, and ADP 0.36 mM. The final volume of reaction mixture in polarographic chamber was 2.0 ml at pH 7.3.

n = 8. Values are mean ± S.E.M.

TABLE VII

RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF PYRUVATE AND SUCCINATE

BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OF 5,5 DIPHENYI-2-THIOHYDANTOIN (DPTH) AND PENTABARBITOL

	State 3 Qo_2 (mmoles O_2/mg P/min			Ratio ADP/O		
Substrate (mM)	Without DPTH	With DPTH		Without DPTH		With DPTH
10 Pyruvate	34.6±2.6	20.3±1.8	(P<0.001)	2.30±0.02	(P<0.05)	2.04±0.05
10 Pyruvate + Pentabarbitol	17.3±0.4[a]	18.3±0.6	(N.S.)	2.22±0.03	(P<0.005)	1.90±0.02
60 Succinate	82.0±3.8	52.5±2.5	(P<0.001)	1.57±0.03	(N.S.)	1.50±0.02
60 Succinate + Pentabarbitol	84.0±1.0	50.3±0.8	(P<0.001)	1.50±0.04	(P<0.05)	1.36±0.02

[a] $P<0.001$ - significance level between pyruvate and pyruvate plus pentabarbitol.

Same reaction mixture as described in Table 6. DPTH (0.6mM).

TABLE VIII

EFFECT OF DIETARY 5,5' DIPHENYL-2-THIOHYDANTOIN (DPTH) ON α GLYCEROPHOSPHATE METABOLISM IN RAT LIVER MITOCHONDRIA.

Treatment		Dihydroxyacetone Phosphate found
		n moles/mg/min
Control	(5)	46.04 ± 1.45
DPTH-Treated*	(5)	0.021± 0.062

*Animals were kept on 0.06% DPTH diet for 24 days and 15 days additional on 0.12% DPTH diet prior to sacrificing. Same reaction mixture as described in Table 4, except that α-glycerophosphate 10 mM, instead of pyruvate was used as substrate.

TABLE IX

EFFECT OF DIETARY 5,5 DIPHENYLTHIOHYDANTOIN (DPTH) ON OXYGEN CONSUMPTION BY RAT TISSUE SLICES WITH GLUCOSE AS SUBSTRATE.

Treatment		Oxygen Consumed
		μmoles O_2/g dry weight/minute
Control	(7)	4.21 ± 0.29
		(P < 0.001)
DPTH-Treated	(9)*	2.51 ± 0.15

*Animals were kept on 0.10% DPTH diet for two weeks.
Slices weighing 60 to 80 mg were then placed in Warburg flasks containing 3 ml of Krebs-Ringer phosphate solution and 10mM glucose as substrate. The pH of the solutions was adjusted with phosphate buffer to 7.3. Flasks were placed in 37°C water bath, shaken and gassed with 100% oxygen for 5 min. Side arms were then closed and measurement of oxygen consumption was commenced 10 min after flasks were placed in the water bath. The oxygen consumption was resumed for one hour.

TABLE X

EFFECT OF DIETARY 5,5 DIPHENYL-2-THIOHYDANTOIN ON LIVER GLUCONEOGENIC ENZYMES ACTIVITY

Enzyme Activity	Control	DPTH* Treated	
	μmoles/g liver/Min.		
Pyruvate carboxylase (μmoles of $^{14}CO_2$ fixed)	7.64 ± 0.60	2.83 ± 0.21	(P < 0.001)
Phosphoenolpyruvate carboxykinase (μmoles of $^{14}CO_2$ fixed)	1.57 ± 0.12	0.73 ± 0.11	(P < 0.001)
Glucose-6-phosphatase (μmoles of Pi liberated)	16.1 ± 0.51	11.80 ± 0.17	(P < 0.001)
Fructose-1,6-diphosphatase (μmoles NADP reduced)	6.43 ± 0.89	4.66 ± 0.16	(P < 0.05)

N = 4

*Animals were fed 0.1% DPTH diet for two weeks.

TABLE XI

ACTIVITIES OF HEPATIC GLUCONEOGENIC ENZYMES IN ACETYLSALICYLIC ACID-FED AND CONTROL RATS*

Enzyme	Control	Acetylsalicylic Acid-Fed	P
Pyruvate carboxylase (μmoles CO_2 fixed/g liver wet wt/min)	8.21±0.90+	4.00±0.26	0.01
Phosphoenolpyruvate carboxykinase (μmoles/CO_2 fixed/g liver wet wt/min)	4.29±0.36	1.76±0.27	0.01
Glucose-6-phosphatase (μmoles Pi liberated/ g liver wet wt/min)	28.77±2.85	24.23±1.68	N.S.
Fructose-1,6-diphosphatase (μmoles NADP reduced/g liver wet wt/min)	3.34±0.22	3.30±0.15	N.S.

*Both the control and acetylsalicylic acid-fed rats were fasted for the 20 hr prior to sacrifice.

+Each value represents the mean ± S.E.M. of enzyme activities obtained from 5 rats. The P value was calculated by Student's t test.

TABLE XII

ACTIVITIES OF KIDNEY GLUCONEOGENIC ENZYMES IN ACETYLSALICYLIC ACID-FED AND CONTROL RATS*

Enzyme	Control	Acetylsalicylic Acid-Fed	P
Pyruvate carboxylase (μmoles CO_2 fixed/ g kidney wet wt/min)	6.66±0.55+	6.18±0.26	N.S.
Phosphoenolpyruvate carboxykinase (μmoles CO_2 fixed/ g kidney wet wt/min)	2.64±0.06	1.65±0.09	<0.01

*Both the control and acetylsalicylic acid-fed rats were fasted for the 20 hr prior to sacrifice.

+Each value represents the mean ± S.E.M. of enzyme activities obtained from5 rats. The P value was calculated by Student's t test.

TABLE XIII

LIVER GLYCOGEN LEVELS IN ACETYLSALICYLIC ACID-FED AND CONTROL RATS*

Enzymes	Control	Acetylsalicylic Acid-Fed	P+
Liver glycogen (µmoles of glucose liverated/g liver wet wt)	61.0±6.7+	23.7±2.1	<0.01

*Both the control and acetylsalicylic acid-fed rats were fed until sacrificed.

+Each value represents the mean ± S.E.M. of glycogen determinations from 5 rats.